Available online at www.sciencedirect.com
ScienceDirect
European Journal of Pharmaceutics and Biopharmaceutics 67 (2007) 655–666
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

Liposomes for drug delivery to the lungs by nebulization

Marco Zaru [a,b], Spyridon Mourtas [a], Pavlos Klepetsanis [a,c], Anna Maria Fadda [b], Sophia G. Antimisiaris [a,c,*]

[a] Department of Pharmacy, University of Patras, Rio, Greece
[b] Department of Farmaco Chimico Tecnologico, University of Cagliari, Cagliari, Italy
[c] Institute of Chemical Engineering and High-Temperature Processes-FORTH, Patras, Greece

Received 4 December 2006; accepted in revised form 11 April 2007
Available online 18 April 2007

Abstract

Preparation of drug-loaded freeze-dried (FD) liposomes, designed for delivery to lungs after rehydration/nebulization was investigated. Rifampicin (RIF) incorporating multilamelar (MLV) and dried rehydrated vesicles (DRV); composed of phosphatidylcholine (PC), dipalmitoyloglycero-PC (DPPC) or distearoyloglycero-PC (DSPC), containing or not Cholesterol (Chol), were prepared. Vesicles were characterized for encapsulation efficiency (EE%), size distribution, zeta-potential, stability during freeze drying (FD) and nebulization (nebulization efficiency (NE%) and retention of RIF after nebulization (NER%)). Mucoadhesion and toxicity in A549 cells was measured. RIF EE% was not affected by liposome type but lipid composition was important; Synthetic lipid vesicles (DPPC and DSPC) had higher EE% compared to PC. As Chol increased EE% decreased. Freeze drying (FD) had no effect on EE%, however trehalose decreased EE% possibly due to RIF displacement. NER% was highly affected by lipid composition. Results of NE% and NER% for RIF-loaded liposomes show that DSPC/Chol (2:1) is the best composition for RIF delivery in vesicular form to lungs, by nebulization. Mucoadhesion and A549 cell toxicity studies were in line with this conclusion, however if mucoadhesion is required, improvement may be needed.

Keywords: Liposome; Lipid composition; Microparticle; Alveolar delivery; Lungs; Rifampicin; Nebulization; Aerosol; Amphiphilic drug; Mucoadhesion

1. Introduction

For the administration of drugs that are intended to act topically in the lungs, direct deposition to the lungs by some type of aerosol delivery technology [1], as nebulization of liquid solutions, offers the advantage of increased drug localization at the site of action and decreased possibility of side effects compared to systemic administration. If this administration route is combined with the sustained release and/or targeting potential of novel drug delivery systems, the therapeutic advantages are even more pronounced. For such applications, it is important to have background knowledge about the parameters that would influence the stability of the drug-carrier system during the aerolization process, as well as the ability of the system to be aerolized.

Between the various types of drug delivery systems proposed, liposomes have many advantages, as their suitability for lipophilic drugs and the fact that they have potential for prevention of local irritation, increased potency and reduced toxicity [2]. Liposomal aerosols in pulmonary therapy offer the additional advantage of uniform deposition of locally active drugs [2,3]. Between the various available aerosol delivery technologies, nebulizers offer the additional advantage of delivering liposomal drugs without further processing [4–6]. This of course applies; if the liposomal drug (in aqueous dispersion form) has the appropriate stability (especially towards lipid oxidation [which can be solved by addition of proper types of anti-oxidants] or drug

* Corresponding author. Laboratory of Pharmaceutical Technology, Department of Pharmacy, University of Patras, Rio 26510, Greece. Tel.: +30 2610 969332; fax: +30 2610 996302.
E-mail address: santimis@upatras.gr (S.G. Antimisiaris).

0939-6411/$ - see front matter © 2007 Published by Elsevier B.V.
doi:10.1016/j.ejpb.2007.04.005

hydrolysis [which depends on the drug formulated]) in order to provide an adequate shelf-life for the formulation. Nevertheless, if formulation stability is a problem and cannot be solved by addition of stabilizer, it may be required to add additional steps in the whole process, in order to lyophilize the liposomal formulation (for adequate product shelf-life) and re-hydrate the powder before nebulization [7]. In this case, in addition to all other parameters the easiness to rehydrate the powder-lyophilizate produced should also be considered. Another possibility, which will not be considered in this investigation, would be the usage of a dry powder nebuliser.

In previous studies, the nebulization efficiency of liposomal systems has been found to be influenced by many parameters, as the lipids used for liposome formation as well as the method of vesicle preparation [8] and the concentration of the lipid dispersion nebulised [8–12]. It is known that liposomes might suffer damage during nebulization because of the high shear forces developing in the nebulizers [8,9] and/or the effect of the droplet impact on baffles on the fluid properties of liposomes [13]. Vesicle size, nebulizer operating conditions and lipid composition have been found to influence the stability of liposomes during nebulization. However, although several studies have been performed with different drugs, the effect of the interactions taking place between the drug and the lipid membrane and their influence on vesicle nebulization has not been clarified. As the mechanism of drug release from liposomal formulations of amphiphilic drugs is more complex (compared to hydrophilic drugs), we chose to use rifampicin (RIF) an amphiphilic drug, in this study. The complexity mentioned above, rises from the fact that in addition to being encapsulated in vesicle aqueous compartments (as much as drug solubility permits), an ampliphilic drug will most possibly also interact with (and be incorporated in) the lipid membrane. Furthermore it may also alter the membrane properties [14]. Nevertheless in addition to its amphiphilic properties, RIF was selected also because it is a first choice drug for tuberculosis treatment [15,16] and resistance to RIF can develop rapidly [16]. Thereby, there is a therapeutic rational for delivering RIF with liposomes, in order to passively target alveolar macrophages where a large number of tubercule bacilli harbour [17]. For these reasons, several types of novel-drug delivery devices have been proposed and characterized for RIF administration, in order to maximize the therapeutic and minimize the toxic and/or side effects [18–22]. From these previous studies, some information about incorporation of this drug in some liposome types is available and can be used for comparison with the results obtained herein.

In this study two different types of RIF liposomal formulations, using nine different lipid compositions were prepared and nebulized. The effect of liposome type (multilamellar vesicles [MLV] and dried rehydrated vesicles [DRV] were studied) and lipid composition on initial drug incorporation, nebulization ability and stability during nebulization were investigated. Calorimetric studies were performed in order to have definite information about the interaction between RIF and lipid membranes, although some information is available in the literature [23]. As explained above, depending on the drug used, it may be needed to freeze dry the liposomal formulation to obtain adequate shelf-life, and rehydrate before nebulization. Since it is known that depending on the type and extent of interactions between liposome incorporated molecules and lipid-membrane components, cryoprotectants, as trehalose, may increase or decrease the amount of the incorporated molecule retained in vesicles [24] the effect of different amount of trehalose (cryoprotectant) on the retention of RIF in vesicles during freeze drying was studied. Additionally, the stability during nebulization of the resulting -after rehydration- RIF-liposomes was evaluated. To our knowledge, such studies have never been performed.

Although for the specific application to target liposomal RIF to alveolar macrophages the liposome formulation may not benefit from increased mucoadhesive ability (a point that is under debate), in other applications mucoadhesive properties of liposomal formulations intended for delivery to the lungs are very important, for prolonged retention and slow release of drugs at the site [25,26]. This is why we evaluated the mucoadhesive properties of (some of) the liposomes prepared, in order to provide some knowledge about if and how the liposomal composition (of liposomal drug formulations) influences these properties.

Additionally, the toxicity of (some of) the liposomal formulations prepared towards an alveolar epithelial cell line was evaluated, and also compared with that induced by equivalent concentrations of the free drug (in solution) measured under identical experimental conditions.

2. Materials and methods

Egg phosphatidylocholine (PC), Dipalmitoyloglycerophosphatidyl-choline (DPPC) and Distearoyloglycerophosphatidyl-choline (DSPC) were purchased from Lipoid, Gmbh (Ludwigshafen, Germany) and were demonstrated to give single spots on TLC (New 1990). Cholesterol (Chol), Rifampicin, mucin, Bradford reagent (Coomasie® dye binding protein assay), diphenylhexatriene (DPH) and all other reagents used were of analytical grade and purchased from Sigma-Aldrich OM, Athens, Greece.

The A549 epithelial alveolar cell line (passage 31) was a kind gift from Dr. Ben Forbes (School of Pharmacy, Kings College, London). All media used for cell growth and handling were purchased from Biochrom (Berlin, Germany), and were of cell culture grade.

2.1. Liposome preparation

Multilamellar vesicles (MLV) were prepared by the thin film hydration method [27]. In brief, the lipid (5 mg of total lipid) (PC, DPPC or DSPC) and the drug (2 mg added, as

solution in methanol) after being mixed (or not) with cholesterol (Chol) [lipid/Chol 2:1 or 1:1 mol/mol ratio] were dissolved in a chloroform/methanol (2/1, v/v) mixture and the final lipid solution was placed in a round bottom flask that was subsequently connected to a rotor evaporator in order for the organic solvents to evaporate, resulting in the formation of a thin film of lipids. For complete removal of organic solvents the film was dried under nitrogen for 3–5 min. The lipid film was hydrated with 1 mL of Tris buffer pH 7.40, (5mM tris) containing 20 mM NaCl. The resulting liposome dispersions were placed in a bath-type sonicator for 15 min.

For DRV preparation, the dehydration–rehydration procedure [28] was used. In brief, empty small unilamellar vesicle (SUV) liposomes were initially prepared by probe sonication of MLV dispersions (prepared as described above but without drug addition) using a vibra cell sonicator (Sonics and Materials, UK) equipped with a tapered micro-tip, and by applying at least two 10 min cycles, until the initially turbid liposomal suspension was well clarified. The Ti-fragments and any multilamellar vesicles or liposomal aggregates were removed by centrifugation at 10,000*g* for 15 min. Subsequently, 1 mL of SUV dispersion was mixed with 2 mL of RIF solution (containing 2 mg of RIF) and the mixture was freeze dried. Upon controlled rate rehydration of the dried materials obtained by freeze drying, as described previously [28], multilamellar dehydrated–rehydrated vesicles (DRV) were generated.

Following their formation, liposome suspensions (MLV and DRV) were left to stand for 2 h at a temperature above the transition temperature of the lipids used in each case, in order to correct any structural defects.

Liposomes were separated from non-entrapped drug by centrifugation (Heraeus Biofuge, Germany); at 15,000 rpm for 40-min. At least two centrifugations were performed.

2.2. Liposome encapsulation efficiency determination

For the calculation of RIF encapsulation efficiency of liposomes, the drug as well as the lipid content of each liposome preparation was measured, as described below. For rifampicin concentration in liposomes, a 200 μL sample of each liposome dispersion was completely dissolved in 5 mL of methanol and the rifampicin concentration was calculated by the optical density of the methanol solution at 485 nm, according to a calibration curve constructed by standard solutions of rifampicin in methanol (linear in the 2–50 ppm range).

For the measurement of phospholipid concentration of RIF-loaded liposomes the DPH fluorescence method was initially used [29], due to the fact that one of RIF absorption maxima is exactly in the same wavelength area (485 nm) used for measurement of lipid concentration in most routine analytical methods (as the Stewart assay [30] or enzymatic assay for phospholipids [31]). Briefly, DPH was dissolved in tetrahydrofuran (THF) to give a concentration of 3 mM, and from this stock solution a 2 μM DPH solution in millipore water was prepared fresh daily. 200 μL of liposome dispersion were mixed with 3 mL of the fresh DPH aqueous solution, the samples were wrapped in aluminum foil and incubated at 37 °C for 30 min and then rigorously vortexed in the absence of direct light. Fluorescence measurements were performed using a Shimatzu spectrofluorometer (excitation 365 nm and emission 420 nm, high sensitivity, 5 nm slits).

After establishing (Fig. 1) that results obtained by this fluorescent assay and those obtained by the Stewart assay are statistically equal in the 0–200 ug/mL phospholipid concentration range (due to the fact that the amount of RIF in the diluted liposomes, is very low, and thus it practically does not interfere with the phospholipid concentration measurement), the colorimetric assay [30] was used to measure lipid concentrations routinely.

2.3. Calorimetric studies

Differential scanning calorimetry was used to determine the phase transition temperatures of fully hydrated phospholipid samples, containing or not different amounts of RIF. The main transition (at Tm) represents the rippled gel-to-liquid crystalline phase transition. The transition temperatures correspond to the peaks of the endotherms during the heating scans. A Perkin Elmer calorimeter (Perkin Elmer Diamond DSC) at a scan rate of 10 °C/h was used. The samples consisted of a known mass (5–10 mg) of 100 mM phospholipid solution in the tris buffer the liposomes were initially prepared in. Sample runs were repeated at least 3 times on two or more different batches to ensure reproducibility. The standard deviation was lower than 0.05 °C. The analysis of the HS-DSC endotherms was conducted using Microcal's Origin, version 6.

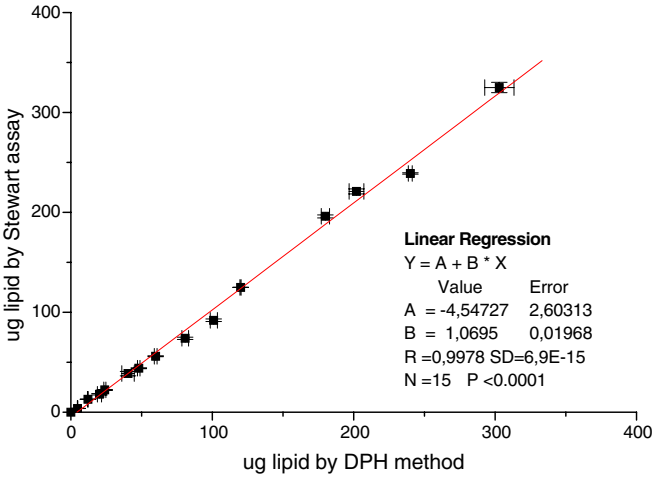

Fig. 1. Comparison between lipid concentration values measured by the DPH and Stewart assay, for RIF-loaded liposome samples. The linear regression parameters calculated are presented in the figure insert.

2.4. Liposome nebulization ability

Liposome aerosols were generated using an efficient high-output continuous-flow Medeljet nebuliser (0.38 L/min), driven by a Medel Z17 compressor operated at 11 L/min. A volume of 3 mL was used for the nebulisation. The aerosols containing RIF-liposomes were collected in a buffer solution using a home-made 2-stage glass impinger (as shown in Fig. 2). The impinger device was utilized with the collecting flask containing 3 mL of buffer to which the aerosol was introduced through a calibrated glass tube and critical orifice delivering the jet of aerosol 5 mm above the bottom of the flask. After aerosolization (which ended after approximately 10 min) the RIF and the lipid contained in the impinger were assayed in order to evaluate the total output and the effect of nebulization on liposomes (drug leakage). Sample dilution was account for after measuring the exact volume of dispersion collected. The nebulization efficiency (NE%) was calculated considering the drug/lipid (D/L) collected on the impinger and was expressed as a percentage of the initial D/L submitted to nebulization:

$$\%\ \mathrm{NE}\% = \frac{\text{Aerolised D/L(collected-in-flask)}}{\text{Initial D/L(placed-in-nebuliser)}} \times 100$$

2.5. Evaluation of Rifampicin retention in liposome

The membrane integrity of liposomes after freeze drying or nebulization, or both, was evaluated. For this, liposome dispersions (1 mL) of known D/L mol/mol ratios, were freeze dried in absence or in presence of trehalose (10, 30 and 100 mM final concentration of trehalose in the liposome dispersions) and rehydrated, by one step addition of 1 mL H_2O. Trehalose is one of the cryoprotectants used for cryopreservation of liposomes or even cell membranes with demonstrated good preservation efficiency [32]. The percentage of drug entrapped in the vesicles was then calculated, after separating the vesicle from released drug and measuring the drug and lipid content of the samples, as described in detail above. Following freeze-drying, and rehydration the samples were nebulized, as described above (in some cases samples were nebulized without being freeze dried) and after collection of the nebulized liposomal dispersion and measurement of the nebulization efficiency (NE%) (as described above) the nebulized liposomes were separated from any RIF that was released from the vesicles during the process (by centrifugation, as described above) and finally the retention of RIF in nebulized vesicles (NER%) was calculated by the following formula:

$$\mathrm{NER}\% = \frac{\text{Aerolised \& Purified D/L(collected-in-flask)}}{\text{Aerolised D/L(collected-in-flask)}} \times 100$$

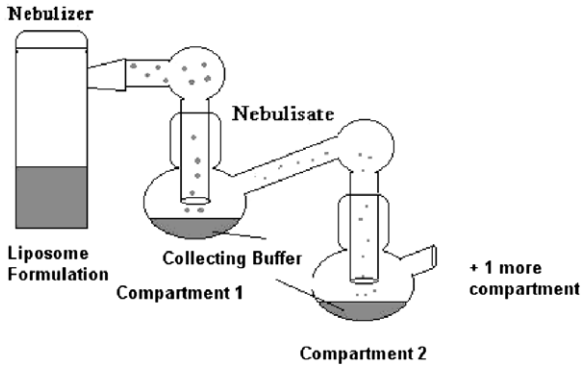

Fig. 2. Apparatus used for collection of the nebulized formulations of liposomes. In all cases, most of the nebilized material was collected in a total of 5 mL of buffer, pH 7.40.

2.6. Measurement of liposome size and surface charge

The size distribution (Mean diameter and Polydispersity Index) and ζ-potential of some of the liposome dispersions were measured by dynamic light scattering (DLS) and laser Doppler electrophoresis (LDE), respectively, on a Nano-ZS (Nanoseries, Malvern Instruments), which enabled the mass distribution of particle size as well as the electrophoretic mobility to be obtained. Measurements were made at 25 °C with a fixed angle of 173°. Sizes quoted are the *z*-average mean (dz) for the liposomal hydrodynamic diameter (nm). Calculation of ζ-potential (mV) was done by the instrument (from electrophoretic mobility).

2.7. Mucoadhesive studies – adsorption of mucin on liposomes

The adsorption of mucin on vesicle surface was used as a method to assess mucoadhesive properties of the liposomes prepared. For this, 1 mL of mucin aqueous solution (0.5 mg/mL) was mixed (vortexed) with 1 mL of each liposome dispersion (the concentration of lipid in these dispersions was fixed at 2 mg/mL) at room temperature. Then, the dispersions were centrifuged at 15,000 rpm for 30 min, and the supernatant was used for the measurement of free mucin. PC/Chol and DSPC/Chol liposomes were studied. Additionally chitosan microparticles were used as positive controls (because they are known to have good mucoadhesive properties). Also the effect of adding negative charge on liposomes – by including 10 mol% of phosphatidylglycerol (a negatively charged lipid) in their membranes – on vesicle mucoadhesive properties was evaluated.

The Bradford colorimetric method [33] was used to determine free mucin concentration in order to assess the amount of mucin adsorbed on the liposomes. A mucin calibration curve was also prepared by measuring mucin standard solutions (0.25, 0.5, 0.75, 1 and 1.25 mg/mL). For all samples (of known and unknown mucin concentration) after adding Bradford reagent, the samples were incubated at 37 °C for 20 min and then the absorbance at 595 nm was measured (Shimatzu UV-1205 spectrophotometer). The

mucin content of the samples was calculated from the standard calibration curve.

2.8. Cell culture studies

Human A549 alveolar cells (at passage 31) were grown as monolayers in 35 mm tissue culture dishes incubated in 100% humidity and 5% CO_2 at 37 °C. HAM'S medium containing 365 mg/L L-glutamine, supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin was used as growth media. The cells that form monolayers were harvested with trypsin (0.25%) centrifuged at low speed (1600*g*, 4 min), re suspended in fresh medium and plated at a concentration of 2×10^5 cells/dish. The cells were grown to confluence on tissue culture dishes after 3–4 days.

2.8.1. RIF and liposomal RIF cyctotoxicity assessment – MTT assay

The effect of empty and RIF-loaded liposomes on the viability of cells was determined by [3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] MTT assay [34]. The dye is reduced in mitochondria by succinic dehydrogenase to an insoluble violet formazan product. A549 cells (10^5 cells/well) were cultured on 24-well plates with 500 μL of medium. The cells were incubated for 24 h with and without the tested compounds. Then 50 μL of MTT (5 mg/mL in PBS) were added to each well and after 2 h the formazan crystals formed were dissolved in DMSO. Absorbance at 580 nm was measured with a plate reader-spectrophotometer.

On the basis of this assay % cell viability values were obtained in three independent experiments for each formulation.

2.9. Statistical treatment of experimental results

For the statistical evaluation of differences between results, the paired *t*-test, to check the significance between EE%, NE% and NER% values calculated for the different liposome types and compositions, was used. In all cases, a probability value of less than 0.05 was considered to be significant.

3. Results

3.1. Physicochemical characteristics of RIF-loaded MLV and DRV liposomes and stability during nebulization

In Table 1 the RIF trapping efficiency (percent of drug entrapped) in MLV and DRV liposomes is presented, and the amount of drug entrapped in vesicles per lipid, for all the preparations formulated, is seen in Fig. 3. RIF encapsulation ranges between 20 and 60 mmol RIF/mol lipid. From Table 1, it is obvious that liposome type does not have any effect on the percent of RIF entrapped in liposomes (no significant difference between MLV and DRV liposomes with the same lipid composition). Nevertheless, lipid composition is a very important determinant of RIF encapsulation. As seen in Fig. 3, for both types of vesicles (MLV-top graph and DRV-bottom graph) the amount of RIF entrapped in liposomes per mol lipid increases signif-

Table 1
Trapping efficiency, mean diameter and ζ-potential values of RIF encapsulating MLV and DRV liposomes, composed of different phospholipids without or with Chol in their membranes

	Trapping efficiency %		Mean diameter (μm)	ζ-potential (mV)
Lipid composition	MLV	DRV	MLV	MLV
PC	18.6 ± 1.5	20.2 ± 1.1	3.36 ± 0.20 [P[a]: 0.451 ± 0.110]	−2.19 ± 0.79
PC/Chol (2:1)	14.3 ± 2.1	14.7 ± 2.7	5.9 ± 1.2 [P: 0.374 ± 0.042] Empty: 5.30 ± 0.91[b] [P: 0.29 ± 0.10]	+2.65 ± 0.75
PC/Chol (1:1)	10.2 ± 2.4	12.4 ± 2.4	NM	NM
DPPC	44.5 ± 3.4	45.7 ± 1.4	8.5 ± 1.0 [P: 0.26 ± 0.20]	−1.97 ± 0.51
DPPC/Chol (2:1)	28.6 ± 4.7	31.5 ± 3.0	NM	NM
DPPC/Chol (1:1)	24.1 ± 2.7	27.6 ± 5.9	NM	NM
DSPC	37.7 ± 1.8	40.3 ± 1.4	8.01 ± 0.11 [P: 0.83 ± 0.12]	−0.246 ± 0.318
DSPC/Chol (2:1)	31.9 ± 4.2	35.5 ± 1.0	10.9 ± 1.5 [P: 0.179 ± 0.153] Empty: 7.1 ± 1.6[a] [P: 0.927 ± 0.769]	−0.408 ± 1.49
DSPC/Chol (1:1)	28.4 ± 1.4	31.5 ± 1.8	NM	NM

Each value is the mean calculated from at least 3 separate preparations and standard deviation of mean is presented.
[a] P, Polydispersity Index for the measurements (mean ± SD).
[b] Empty = The same liposomes without RIF.

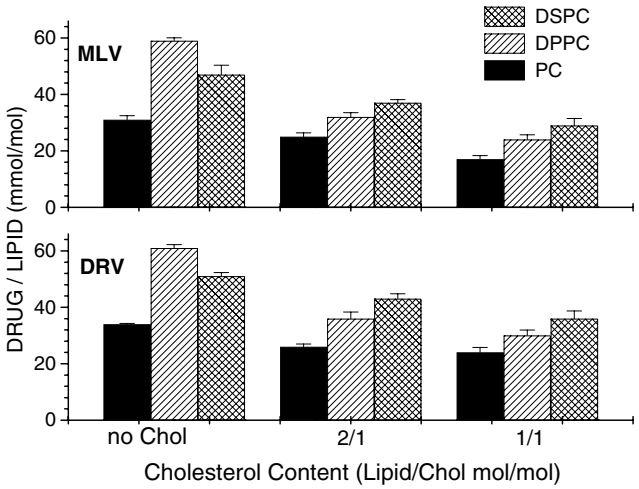

Fig. 3. Rifampicin encapsulation efficiency (D/L [Drug/lipid] mmol/mol) in MLV and DRV liposomes composed of PC, DPPC or DSPC, without or with Chol included in the membranes (2:1 or 1:1 Lipid/Chol mol/mol). Each value is the mean calculated from at least three separate preparations and standard deviation of mean is presented as error bars.

icantly ($P < 0.05$) when liposomes are formed from saturated lipids (DPPC < DSPC in most cases) compared to PC-based liposomes. Inclusion of Chol in the lipid bilayers, always results in a decrease of RIF EE%, which is logical due to the lipophilic nature of RIF (the drug is displaced from the bilayers by Chol).

From the results of mean diameter measurements (Table 1) it is seen that all vesicle-types are considerably large with mean diameters ranging between 3.36 and 10.90 μm. In most cases the polydispersity indexes measured are low indicating that the vesicles are monodisperse. Addition of Chol in the lipid membranes of liposomes always results in increased vesicle size. However, the large size of the liposomes is also partly due to the presence of RIF. This is proven by the fact that RIF entrapping PC vesicles are considerably smaller compared to DPPC and DSPC ones, which also incorporate significantly more RIF molecules per lipid. Additionally, empty PC/Chol and DSPC/Chol liposomes prepared under identical conditions (with the corresponding RIF-entrapping vesicles) are significantly smaller compared to the vesicles that incorporate the drug. This clearly indicates that RIF molecules intercalate into the lipid bilayers, as anticipated by its lipophilic nature. The bulky size of RIF is thus responsible for the increased mean diameter of the RIF-liposomes compared to empty. Such increases were not measured previously when other lipophilic drugs were incorporated in liposomes [14], however those drugs were smaller.

The fact that RIF molecules interact with phospholipids in the liposome membrane has been proven previously by NMR and DSC [23] studies, however DSC studies were also performed. As observed in Fig. 4, and Table 2, the disappearance of the pre-transition peak and the presence of a peak with a lower Tm compared to that measured for pure DPPC (liposomes dispersed in TBS buffer, pH 7.40) when RIF is added in the liposomes, suggests that the neighboring phospholipids are packed less tightly in the RIF containing vesicles. This could be achieved by their interaction with RIF molecules through hydrogen bonds. The high transition temperature of DPPC liposomes (compared to literature values [usually around 41 °C]) is due to the fact that they were dispersed in buffer. The effect on the thermotropic properties of DPPC membranes is enhanced when more RIF is incorporated in the membranes. Even in the DPPC/Chol (2:1) liposomes, in which Tm is practically abolished, because of the addition and interaction of cholesterol with the membrane, addition of RIF seems to influence the thermograph, proving that a portion of

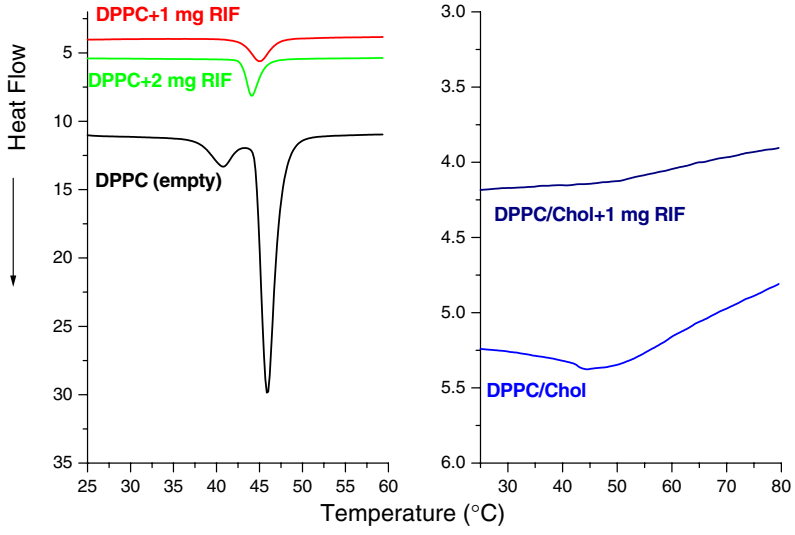

Fig. 4. Influence of rifampicin on the heat differential flow of the phase transitions of DPPC and DPPC/Chol (2:1) containing bilayers. The lipid content in the calorimetric pan ranged from 4.8 to 11 mg and the hydration media was a 5 mM Tris buffer (pH 7.40) made isotonic with NaCl. The calorimetric curves presented corresponding to the different lipid mixtures, in the absence and in the presence of RIF (as shown in the graph), were digitally recorded at a scan rate of 10 °C/min.

Table 2
Effect of rifampicin on the thermodynamic parameters of aqueous dispersions of DPPC lipidic systems

Liposome composition	T_m (°C) [onset-end]	ΔH (J/g)
DPPC	*Pre transition:* 40.61 [37.82–42.47]	2.199
	Main transition: 45.94 [44.57–47.66]	19.02
DPPC + 1 mg RIF	*(one transition peak):* 45.06 [42.82–47.21]	6.20
DPPC + 2 mg RIF	*(one transition peak):* 44.04 [42.91–45.76]	4.84

the liposome-associated RIF is in the lipid membrane, although a substantial part of it has been displaced by Chol molecules (as indicated by the decreased RIF EE%). This suggestion correlates well with the 28.6% trapping efficiency measured for the Chol-containing liposomes (Table 1), since such an EE% value could not be achieved if the drug was only encapsulated in the aqueous compartment of the vesicles.

Concerning vesicle ζ-potential, the values measured for all types of vesicles were very low (Table 1), indicating that the lipid membranes are not charged, as expected (since no charged lipid is present in the lipid bilayer), and this was not affected by the presence of the drug in the membranes.

In Table 3, the NE% and NER% values calculated after the liposome dispersions were subjected to nebulization, are presented. In most cases the materials nebulized (NE%) were higher than 70 percent of initial materials placed in the nebulizer, and this is practically not affected by liposome type or lipid composition, with the exception of 50 mol%-Chol-containing DPPC and DSPC liposomes, for which NE% was <70% (and especially for the DSPC/Chol (1:1) liposomes, very low (32–43%)). This finding suggests that very rigid liposomes cannot be efficiently nebulized.

As observed in Table 3, the main parameter determining the percent of RIF retained in the nebulized liposomes

Table 3
Nebulization efficiency [NE%] (percent of material that was nebulized) and percent of RIF retained in vesicles in the nebulized fraction [NER%], for liposomes with different lipid compositions

Lipid composition	MLV		DRV	
	NE%	NER%	NE%	NER%
PC	78 (1.7)	15.31 (1.2)	81 (4.6)	16.3 (4.9)
PC/Chol (2:1)	89 (5.9)	34.5 (2.3)	92.0 (3.4)	21 (1.0)
PC/Chol (1:1)	77.2 (9.4)	41.1 (8.4)	86.4 (4.4)	28.5 (4.3)
DPPC	70.3 (7.1)	35.3 (4.0)	72.3 (5.3)	28.2 (3.9)
DPPC/Chol (2:1)	70.3 (9.2)	57.8 (6.2)	72.2 (4.2)	77.0 (8.4)
DPPC/Chol (1:1)	65 (4.5)	16.1 (7.9)	68.1 (5.4)	49.9 (6.1)
DSPC	73.0 (7.1)	58.0 (5.3)	73.2 (7.3)	65.5 (7.2)
DSPC/Chol (2:1)	62.4 (6.4)	69.3 (5.1)	63.1 (9.0)	71.3 (4.6)
DSPC/Chol (1:1)	42.7 (7.1)	67.5 (8.2)	32.2 (2.1)	74.2 (4.5)

In all cases the vesicles were nebulized immediately after preparation (and separation from non-incorporated drug). Each value is the mean calculated from at least 3 separate preparations and standard deviation of mean is presented in parentheses.

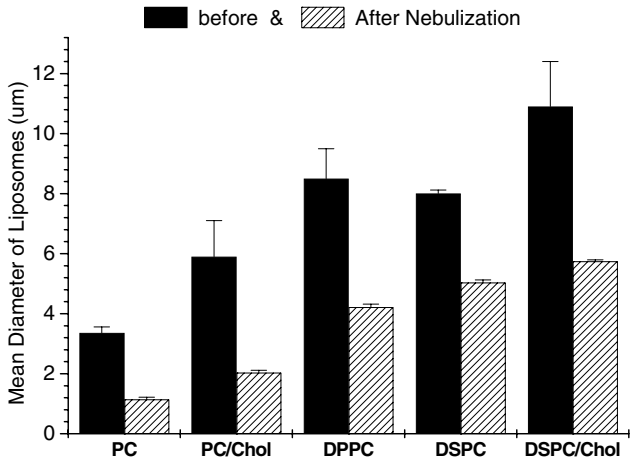

Fig. 5. Comparison of mean diameter (μm) of some RIF-loaded liposomes before and after they have been subjected to nebulization. For each sample at least three formulations were measured (5 measurement per formulation) and the mean diameter (μm) is presented as well as standard deviation of mean (as error bar).

(NER%), is the lipid composition of vesicles. Indeed, moving from PC (that forms liquid membranes) to DPPC and furthermore to DSPC (that form more rigid membranes) vesicle stability and as a consequence to this, also RIF retention in vesicles, increases substantially. The effect of Chol (addition in lipid membranes) on the NER% measured, is different for each lipid studied; in the case of PC-based liposomes as Chol percent increases, NER% also increases, while for DSPC-based liposomes Chol addition in the vesicles has practically no effect on NER% (which is always high due to the rigidity of the DSPC liposomes). However, for the DPPC-based liposomes although NER% is significantly increased when 33 mol% of Chol is included in the vesicle membrane, addition of more Chol in the vesicle bilayers results in a decrease of NER%.

The size of (some of) the vesicles nebulized was also measured after nebulization (and separation from any released drug). As seen in Fig. 5, in all cases nebulization effect vesicle size considerably. Indeed, vesicle mean diameters were reduced by approximately 50%.

3.2. Stability of RIF-loaded liposomes during freeze drying – effect of trehalose

As observed in Table 4, when the vesicles were freeze dried in absence of trehalose the amount of RIF retained in vesicle was equal to that initially entrapped (by comparison with the values presented in Fig. 3). However, when the vesicles were freeze dried in presence of increasing amounts of trehalose, the per cent of RIF retained in the vesicles after freeze drying gradually slightly decreased (as trehalose concentration increased). In fact, when freeze drying was carried out in presence of 100 mM trehalose the decrease of RIF retention in DPPC/Chol and DSPC/Chol liposomes was significant. On the other hand, the quality of powder produced, in terms of easiness of rehy-

Table 4
RIF encapsulation efficiency (mmol RIF/mol lipid) of MLV and DRV liposomes composed of PC, DPPC or DSPC, after freeze drying in presence or not of various trehalose concentrations (0–100 mM)

	Trehalose (mM)	Drug/lipid (mmol/mol)	
		MLV	DRV
PC/Chol (2:1)	0	24.4 (1.4)	25.12 (0.86)
	10	**24.3 (1.1)**	**24.2 (0.63)**
	30	22.16 (0.96)	22.56 (0.80)
	100	22.38 (0.57)	20.33 (0.57)
DPPC/Chol (2:1)	0	32 (3.0)	36.2 (1.7)
	10	**30.8 (1.2)**	**31.8 (3.3)**
	30	27.12 (2.0)	26.2 (1.0)
	100	21.33 (1.2)	18.4 (2.1)
DSPC/Chol (2:1)	0	37.3 (2.1)	43.2 (1.0)
	10	**36.1 (2.6)**	**40.6 (1.5)**
	30	34.9 (2.6)	37.7 (3.3)
	100	31.1 (1.2)	27.2 (1.1)

Liposomes always contain Chol in their membranes at 2:1 mol/mol (Lipid/Chol). Each value is the mean calculated from at least 3 separate preparations and standard deviation of mean is presented in parentheses. For values in bold, please refer to text.

dration, was significantly improved (compared to that produced in absence of trehalose) when freeze drying was carried out in presence of trehalose (even the lowest trehalose concentration used).

3.3. Nebulization of freeze-dried (FD) RIF-loaded liposomes

Freeze dried (FD) RIF-loaded liposomes (in absence or presence of trehalose) were subjected to aerolization (after being rehydrated) and NE% - NER% were measured. As presented in Table 5 it is obvious that NE% of FD-rehydrated liposomes is not affected by liposome type, but only lipid composition (i.e. also observed in the case of non-freeze dried liposomes, Table 3). Indeed NE% is significantly lower in the case of rigid liposomes (DPPC/Chol and DSPC/Chol, which behave similarly) compared to the PC/Chol ones. Oppositely with what was observed for RIF retention after freeze drying (Table 3), the amount of trehalose used during FD had no effect on NE% (the differences are not statistically significant). From the NER% values calculated (Table 5) it is obvious that regardless of liposome type (MLV and DRV liposomes behave similarly) the stability of liposomes during nebulization is determined by their lipid composition. Indeed NER% values increase in the order PC/Chol <DPPC/Chol <DSPC/Chol.

The most important aspect when nebulizing liposomal drugs (when liposomal retention of the drug is required for therapeutic purposes) is the final amount of drug retained in the vesicles after freeze drying and nebulization (if a freeze drying step is unavoidable). As (calculated and) presented in Fig. 6, this final outcome is substantially higher for the DSPC/Chol 2:1, liposomes compared to all the other lipid compositions studied, if a freeze-drying step is required (cases 2 & 3 in Fig. 6). If freeze drying is not necessary (case 1 in Fig. 6) then the final outcome of the DPPC/Chol liposomes is similar with that of the DSPC/Chol ones (for both MLV and DRV liposomes).

Table 5
Nebulization efficiency [NE%] (percent of material that was nebulized) and percent of RIF retained in vesicles in the nebulized fraction [NER%], for liposomes with different lipid compositions

	Trehalose (mM)	MLV		DRV	
		NE%	NER%	NE%	NER%
PC/Chol (2:1)	0	72.7 (3.5)	29.0 (3.1)	84.1 (9.5)	18.3 (4.5)
	10	**81.3 (2.5)**	**16.1 (2.9)**	**59.3 (9.3)**	**11.4 (2.5)**
	30	83.2 (3.1)	13.7 (1.5)	73.7 (5.8)	13.7 (1.5)
	100	84.7 (3.5)	11.6 (2.1)	77.0 (8.3)	10.2 (3.0)
DPPC/ Chol (2:1)	0	50.7 (3.0)	32.6 (3.8)	65.6 (5.1)	32.7 (3.8)
	10	**54.6 (2.5)**	**28.3 (3.1)**	**60.7 (4.0)**	**31.0 (2.6)**
	30	61.7 (2.6)	22.0 (2.1)	54.3 (5.1)	25.1 (2.5)
	100	57.7 (1.5)	20.6 (2.5)	57.7 (1.5)	21.3 (1.6)
DSPC/Chol (2:1)	0	58.3 (5.7)	68.3 (1.5)	58.3 (5.7)	68.6 (1.5)
	10	**59.7 (6.4)**	**59.4 (3.8)**	**57.3 (2.5)**	**62.3 (4.7)**
	30	56.1 (7.2)	56.3 (1.6)	59.1 (7.5)	60.7 (4.7)
	100	54.0 (5.3)	49.2 (2.6)	57.7 (3.2)	59.0 (3.1)

In all cases the vesicles were nebulized after being freeze dried in presence or absence of various trehalose concentrations (0–100 mM) and rehydrated. Liposomes always contain Chol in their membranes at 2:1 mol/mol (Lipid/Chol). Each value is the mean calculated from at least 3 separate preparations and standard deviation of mean is presented in parentheses.
For values in bold, please refer to text.

3.4. Cell toxicity and RIF uptake studies

The viability of A549 cells was estimated after exposure of the cells for 24 h to different concentrations of RIF-

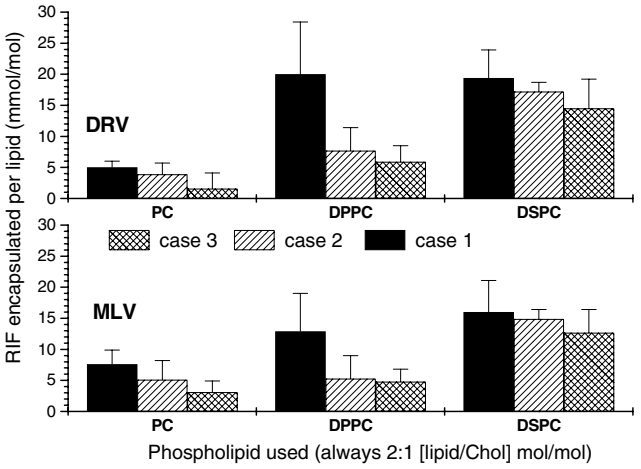

Fig. 6. Comparison of the final outcome (amount of RIF encapsulated in vesicles after nebulization) for 3 different cases of liposome nebulization. Liposomes were composed of PC, DPPC or DSPC (always containing Chol at 2:1 lipid/Chol (mol/mol)). Case 1: liposomes were nebulized immediately after preparation (and separation of non-encapsulated drug), case 2: liposomes were nebulised after freeze drying and rehydration (with no trehalose) and case 3: liposomes were nebulized after freeze drying in presence of 10 mM trehalose (conditions that ensure good powder quality) and rehydration. Each value is the mean calculated from at least 3 separate preparations and standard deviation of mean is presented as error bar.

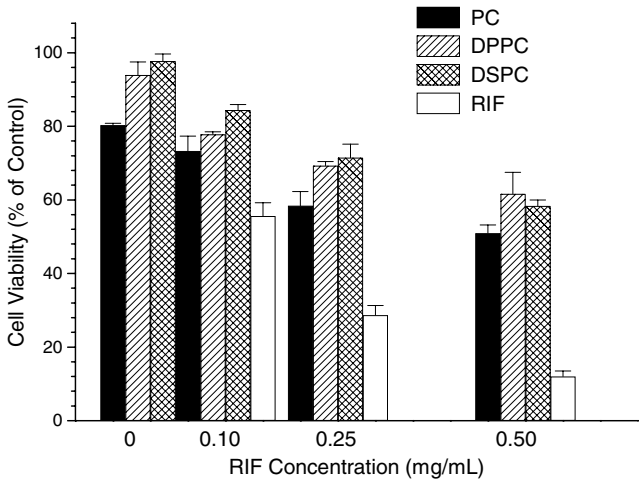

Fig. 7. Viability (% of control) of A549 alveolar cells after they have been incubated for 24 h with various concentrations (0, 0.10, 0.25 and 0.50 mg/mL) of either free RIF, or RIF-incorporated in liposomes. Viability was measured by the MTT assay, as explained in detail in Materials and Methods (2.7). In all cases, liposomes contain Chol in their membranes (at a 2/1 lipid/Chol mol/mol) and the result presented is the mean value calculated (SD of the mean is presented as bar) from at least two different formulations (and 3 different wells /formulation).

loaded liposomes or the same concentration of free RIF (in solution), for comparison. As observed in Fig. 7, when the cells were exposed to empty liposomes (the highest lipid concentrations used in the case of RIF-liposome incubation was selected in each case (i.e. 19, 14 and 13 mg/mL for PC, DPPC and DSPC liposomes, respectively)), cell viability was not affected by DPPC or DSPC empty liposomes, however a slight cell viability reduction was caused by the PC empty liposomes, perhaps due to the higher lipid concentration incubated with the cells in that case. The cytotoxicity induced by free RIF was always substantially higher compared to that induced by RIF-liposomes (containing the same amount of RIF). Whether this is because of a lower uptake of liposomal RIF by the cells (compared to free RIF) or due to a different type of interaction of the cells with free RIF and liposome-associated RIF (that results in different intracellular distribution of RIF rendering the drug less toxic) we do not know. Nevertheless, the lower cytotoxicity of liposomal-RIF, is a considerable advantage of the liposome system.

3.5. Mucoadhesive properties of RIF-loaded liposomes

Mucoadhesive properties of RIF-loaded liposomes were evaluated by measuring mucin adsorption on vesicles. From the results presented in Table 6, it is obvious that mucoadhesive properties of the liposomes tested are low. For comparative reasons it should be mentioned here, that when chitosan microparticles (prepared in our lab for another study), were subjected to the same test more than 90% of mucin was association with the particles. A significant difference was observed between PC/Chol and DSPC/Chol, RIF-loaded liposomes. This could not be attributed to vesicle zeta potential differences between these two liposome types, since both are practically uncharged (Table 1). It is well known that the main mechanism of mucoadhesion is interaction between opposite charged molecules, and therefore a more negative surface charge would reduce mucoadhesive properties. This was indeed demonstrated when negative charge was added on the surface of liposomes (by including 5 mol% of phosphatidyl glycerol in their membranes) and resulted in a marked decrease of the pre cent of mucin adsorpted on the vesicles.

4. Discussion

The preparation of drug-loaded liposomes, that could be used as freeze dried formulations for rapid rehydration–nebulization and delivery of the drug to lung macrophages was investigated. MLV and DRV liposomes were evaluated because these liposome types have particle diameters in the range needed for aerosol delivery to the alveolar region. In order to understand which parameters are important for the preparation of liposomes that can be used for such applications, nine different lipid membrane compositions were evaluated, between which, some form very leaky liquid membranes, as PC without Chol and others very rigid (gel) membranes, as DSPC/Chol 1:1 (mol/mol) liposomes. Rifampicin was used as a drug molecule, first of all because there is a therapeutic rationale for its passive targeting to alveolar macrophages, but also because it is an amphiphilic drug molecule that due to its large size (MW 823 g/mol) and complex structure is considered as a "difficult" drug to encapsulate and especially to retain within liposome vesicles. Thereby, the results and findings of this study can be considered as a basis for the design of liposomal formulations of other drugs intended for administration to the lungs by nebulization.

In general, the trapping values calculated herein for RIF (Table 1) were comparable with those found previously by others [20]. These values reveal that liposome type has no effect on RIF EE% of liposomes. This is logical since RIF is amphiphilic and expected to be incorporated in the lipid membrane of liposomes, thereby differences in entrapped aqueous volumes between the different vesicle types would not result in significant differences for EE% of this drug. However, lipid composition had a high impact on RIF EE%, which was higher in liposomes composed of saturated phospholipids. A similar result was observed by others [35], that measured higher loading capacity for RIF in DPPC/Chol liposomes compare to PC/Chol ones. When Chol was included in liposome membranes, RIF was displaced, as anticipated for an amphiphilic drug, and EE% was decreased (compared to the liposomes without, or with lower percent of Chol in their membranes). In fact, in the case of PC/Chol (1:1) liposomes, the RIF EE% was around 10%, a value that is usual for the entrapment of a hydrophilic drug in vesicles of this size. Thereby, we may conclude that in the vesicles with PC/Chol (1:1) lipid composition all RIF molecules are displaced (by Chol) from the lipid membrane.

On the other hand, the nebulization efficiency NE% for all liposomes studied was practically the same (slight differences measured are not statistically significant) with the exception of the most rigid DSPC/Chol, 1:1, implying that high vesicle rigidity has negative effect on liposome nebulization efficiency. This may be attributed to the higher viscosity of the DSPC/Chol.1:1 liposome dispersion (compared to PC/Chol 1:1 and DPPC/Chol 1:1), since it is known that higher viscosity results in lower nebulization efficiency [10].

Considering liposome stability during nebulization (NER%), Chol-addition in the liposome membranes had a different effect between different vesicles, depending on the lipid membrane rigidity and the amount of Chol added in the membrane. In all cases (for PC, DPPC and DSPC vesicles) the addition of 33 mol% Chol had a positive effect on RIF-NER%, which was significantly increased (compared to NER% of liposomes without Chol in their membrane) especially for PC and DPPC vesicles. However addition of a higher amount of Chol (up to 50 mol% of the lipid content) had a different effect on the retention of RIF during nebulization between the three different lipids studied. Indeed, for the PC/Chol (1:1) liposomes a further increase (compared to PC/Chol 2:1) in RIF-NER% was demonstrated. This can be explained by the fact that (as mentioned above) probably the whole amount (or most) of vesicle-associated RIF is entrapped in the aqueous compartment of this specific vesicle type. No RIF molecules (or perhaps only a very small amount of RIF molecules) are incorporated in the lipid membrane of the vesicles, so the more rigid (compared to PC/Chol 2:1 vesicles) PC/Chol 1:1 membrane can retain the drug better (during the nebulization process). Oppositely in the case of the DPPC vesicle when Chol content was increased from 33 to 50 mol%, a dramatic decline in liposome stability during nebulization was observed, while for the DSPC vesicles no effect on RIF-NER% was noticed. Nevertheless, the later liposomes (DSPC/Chol 1:1) demonstrated very low nebulization ability. Thereby it seems that rich in Chol and rigid liposome membranes are either not able to efficiently retain amphiphilic drugs (as RIF) during nebulization processes (as DPPC/Chol 1:1) or demonstrate very low nebulization efficiency (as DSPC/Chol 1:1). The overall results may in part be determined by the influence of DPPC (or DSPC), Chol and RIF itself on the molecular packing of the lipid bilayers. In other words, as RIF is part of the lipid membrane and also interacts with the lipids (as demonstrated by the DSC study for DPPC), addition of Chol at different amounts will have an impact not only on the bilayer rigidity but also on the interactions between the drug and the membrane components, influencing the stability of drug incorporation in the lipid membrane, and also affecting the retention of the drug in the vesicles during nebulization.

Cholesterol-induced increase of nebulization-induced leakage of drugs from liposomal membranes and decrease of liposome nebulization efficiency have been also reported by others [8,36], but only when 10 mol% of Chol was added in the lipid membranes. This was then attributed to Chol-induced vesicle aggregation. However, 30 mol% Chol addition in liposomes increased liposome nebulization stability [8,36,37] as also demonstrated herein, while the effect of higher Chol-content on liposome behavior during nebulization has not been, to our knowledge, studied before.

The possibility of preparing freeze dried RIF-incorporating liposomes, for improved product shelf-life [7], was additionally evaluated. From a practical point of view when vesicles were freeze dried in presence of 10 mM trehalose, reduction in EE% was minimal (Table 4) while the improvement of the powder quality obtained (easiness to rehydrate) was substantial. In particular, the DSPC/Chol (2:1) RIF-incorporating liposomes demonstrated very high stability (compared to the other lipid compositions) after being freeze dried with this trehalose concentration, rehydrated and nebulized (case 3 in Fig. 6). Nevertheless, from the experiments carried out in presence of various trehalose concentrations some very interesting conclusions can be drawn; indeed it is known that when trehalose is present during freezing and thawing of liposome dispersions, it may displace amphiphilic drugs that are associated with the lipid membrane from the membrane and result in a decrease of encapsulation rather than in an increase of vesicle stability. Thereby, the fact that in DPPC/Chol 2:1 and DSPC/Chol 2:1 liposomes the EE% of RIF is substantially reduced when these vesicle are freeze dried in presence of 100 mM trehalose provides additional proof that RIF is initially associated with the lipid membrane, to some extent. On the other hand trehalose practically has no effect on the RIF- EE% in the case of PC/Chol (2:1) liposomes, proving that either a negligible amount of RIF is associated with the lipid membrane (which is the most possible), or that any amount that is membrane-associated is not displaced by trehalose. Furthermore another interesting observation is that when the trehalose-containing RIF-liposomes are nebulized although nebulization efficiency is not significantly influenced (Table 5), the stability during nebulization (NER%) in the cases of PC/Chol 2:1 and DPPC/Chol 2:1 is significantly affected. This observation indicates that trehalose molecules that remain associated to the membrane after vesicle rehydration and purification from released drug molecules, affect the integrity of the membrane during nebulization. However the higher rigidity DSPC/Chol 2:1 vesicles are practically not affected (Table 6).

Concluding, the results of this study demonstrate that the ability of liposomal formulations to be nebulized and their stability during nebulization is not simple to predict, especially when the drug formulated in liposomes is amphiphilic. In such cases, interactions between the drug and membrane components which affect their packing and as a consequence the rigidity and integrity of the vesicle membrane should also be considered when designing liposomal formulations for delivery to lungs by nebulization. Furthermore, if additional freeze drying steps are required to ensure appropriate product shelf-life, the discpacement of

Table 6
Percent mucin associated with RIF-loaded liposomes after incubation of the liposomes with mucin and centrifugation for separation of liposomes from the non-adsorbed on vesicles mucin fraction

Liposome composition	Mucin adsorbed on liposomes (%) mean value (SD)	ζ-potential (mV)
PC/Chol (2:1)	17.0 ± 8.3	+0.087 ± 0.538
PC/PG/Chol (9:1:5)	7.41 ± 4.4	−22.9 ± 2.1
DSPC/Chol (2/1)	46.2 ± 4.1	+0.927 ± 0.769
DSPC/PG/Chol (9:1:5)	25.1 ± 8.1	−19.9 ± 2.3

Liposome- mucin incubation conditions as well as mucin measurement details are described under materials and methods (Section 2.6). Additionally the ζ-potential values calculated for these vesicles are presented. Triplicate samples were run.

drug molecules by the cryoprotectant used as well as cryoprotectant-induced modulations of lipid membrane properties (that may affect membrane integrity during nebulization) are additional concerns that should be realized.

From a practical point of view, in the case of RIF, the DSPC/Chol 2:1 lipid composition was demonstrated to result in highest nebulization efficiency and integrity. Furthermore, RIF-incorporating liposomes were found to be considerably less cytotoxic towards alveolar epithelial cells compared to free RIF (same RIF concentration in solution), in all cases studied. This may serve as an early indication that liposomal RIF will be less toxic compared to free RIF. Additionally, in line with the relevant bibliography [38,39], the liposomes prepared in this study demonstrated low (compared to mucoadhesive chitosan particles which were used as positive control) association with mucin, as expected for non-charged liposomes. Although it may not apply, in particular for RIF-incorporating liposomes (which are aimed for macrophage uptake) improvement of the mucoadhesive properties of liposomes intended for delivery to the lungs through surface modification may be required for other applications [25,26].

Acknowledgement

EU funded this work under the Marie Curie Early Stage Scholarship Program, Project name: Towards a Euro-PhD in advances drug delivery, Contract No.: MEST-CT-2004-504992.

References

[1] S. Suarez, A.J. Hickey, Nebulizors-aerosol delivery: drug properties affecting aerosol behavior, Respiratory Care 45 (6) (2000) 652–666.
[2] B.E. Gilbert, P.R. Wyde, S.Z. Wilson, R.K. Robins, Aerosol and intraperitoneal administration of ribavarin and ribavarin triacetate: pharmacokinetics and protection of mice against intracerebral infection with influenza A/WSN virus, Antimicrob. Agents Chemother. 35 (7) (1991) 1448–1453.
[3] R. Parthasarathy, B. Gilbert, K. Mehta, Aerosol delivery of liposomal all-trans retinoic acid to the lungs, Cancer Chemother. Pharmacol. 43 (1999) 277–283.
[4] H. Schreier, Liposome aerosols, J. Liposome Res. 2 (1992) 145–184.
[5] M. Saari, M.T. Vidgren, M.O. Koskinen, V.H.M. Turjanmaa, M.M. Neimenen, Pulmonary distribution and clearance of two beclomethasone liposome formulations in healthy volunteers, Int. J. Pharm. 188 (1999) 1–9.
[6] C.F. Lange, R.E.W. Hancock, J. Samuel, W.H. Finlay, In vitro aerosol delivery and regional airway surface liquid concentration of a liposomal cationic peptide, J. Pharm. Sci. 90 (10) (2001) 1647–1657.
[7] Y. Darwis, I.W. Kellaway, Nebulization of rehydrated freeze-dried beclomethasone dipropionate liposomes, Int. J. Pharm. 215 (2001) 113–121.
[8] R.W. Niven, H. Schreier, Nebulization of liposomes. I. Effects of lipid composition, Pharm. Res. 7 (1990) 1127–1133.
[9] K.M.G. Taylor, G. Taylor, I.W. Kellaway, J. Stevens, The stability of liposomes to nebulisation, Int. J. Pharm. 58 (1990) 57–61.
[10] P.A. Bridges, K.M.G. Taylor, An investigation of some of the factors influencing the jet nebulisation of liposomes, Int. J. Pharm. 204 (2000) 69–79.
[11] R.W. Niven, M. Speer, H. Schreier, Nebulization of liposomes. II. The effects of size and modeling of solute release profiles, Pharm. Res. 8 (1991) 217–221.
[12] R.W. Niven, T.M. Carvajal, H. Schreier, Nebulization of liposomes. III. The effects of operating conditions and local environment, Pharm. Res. 9 (1992) 515–520.
[13] WH. Finlay, Chap. 8, in: The Mechanics of Inhaled Pharmaceutical Aerosols: An Introduction, Academic Press, London, 2001.
[14] D. Fatouros, S.G. Antimisiaris, Effect of amphiphilic drugs on the stability and zeta-potential of their liposome formulations: a study with prednisolone, diazepam, and griseofulvin, J. Coll. Int. Sci. 251 (2002) 271–277.
[15] G.L. Mandell, H.A. Sande, Antimicrobial agents, in: G.A. Gilman, A. Goodman (Eds.), The Pharmacological Basis of Therapeutics, seventh ed., Macmillan, New York, 1985, pp. 1202–1205.
[16] H.P. Rang, M.M. Dale, J.M. Rither, Drugs used to treat tuberculosis, in: Pharmacology, fourth ed., Churchill Livingstone, London, 1999, pp. 703–707.
[17] L.E. Bermudez, Use of liposome preparation to treat mycobacterial infections, Immunobiology 191 (1994) 578–583.
[18] C.P. Jain, S.P. Vyas, Preparation and characterizationof niosomes containing rifampicin for lung targeting, J. Microencapsul. 12 (1995) 401–407.
[19] S. Nakhare, S.P. Vyas, Multiple emulsion based systems for prolonged delivery of rifampicin: in vitro and in vivo characterization, Die Pharm. 52 (1997) 224–226.
[20] S.P. Vyas, M.E. Kannan, S. Jain, V. Mishra, P. Singh, Design of liposomal aerosols for improved delivery of rifampicin to alveolar macrophages, Int. J. Pharm. 269 (2004) 37–49.
[21] A. Sharma, R. Pandey, S. Sharma, G.K. Khuller, Chemotherapeutic efficacy of poly (DL-lactide-co-glycolide) nanoparticle encapsulated antitubercular drugs at sub-therapeutic dose against experimental tuberculosis, Int. J. Antimicrobiol. Agents 24 (2004) 599–604.
[22] K. Makino, T. Nakajima, M. Shikamura, F. Ito, S. Ando, C. Kochi, H. Inagawa, G.H. Soma, H. Terada, Efficient intracellular delivery of rifampicin to alveolar macrophages using rifampicin-loaded PLGA microspheres: effects of molecular weight and composition of PLGA on release of rifampicin, Colloids Surf. B: Biointerfaces 36 (2004) 35–42.
[23] M. Bermudez, E. Martinez, M. Mora, M.L. Sagrista, M.A. de Madariaga, Molecular and physicochemical aspects of the interactions of the tuberculostatics ofloxacin and rifampicin with liposomal bilayers: a ^{31}P-NMR and DSC study, Colloids Surfaces A: Physicochem. Eng. Aspects 158 (1999) 59–66.
[24] V. Ntimenou, S. Mourtas, E. Christodoulakis, M. Tsilimbaris, S.G. Antimsiaris, Stability of protein encapsulating DRV liposomes after freeze drying: a study with BSA and t-PA, J. Lipid Res. 16 (2006) 403–416.
[25] J.K. Vasir, K. Tambwekar, S. Garg, Bioadhesive microspheres as a controlled drug delivery system, Int. J. Pharm. 255 (2003) 13–32.

[26] H. Yamamoto, Y. Kuno, S. Sugimoto, H. Takeuchi, Y. Kawashima, Surface-modified PLGA nanosphere with chitosan improved pulmonary delivery of calcitonin by mucoadhesion and opening of the intercellular tight junctions, J. Controlled Release 102 (2005) 373–381.
[27] A.D. Bangham, M.M. Standish, J.C. Watkins, Diffusion of univalent ions across the lamellae of swollen phospholipids, J. Mol. Biol. 13 (1965) 238–252.
[28] C. Kirby, G. Gregoriadis, Dehydration–rehydration vesicles: a simple method for high yield drug entrapment in liposomes, Biotechnology 2 (1984) 979–984.
[29] E. London, W. Feigenson, A convenient and sensitive fluorescence assay for phospholipids vesicles using diphenylhexatriene, Anal. Biochem. 88 (1978) 203–211.
[30] J.C.M. Stewart, Colorimetric determination of phospholipids with ammonium ferrothiocyanate, Anal. Biochem. 104 (1980) 10–14.
[31] H. Grohganz, V. Ziroli, U. Massing, M. Brandl, Quantification of various phosphatidylcholines in liposomes by enzymatic assay, AAPS PharmSciTech 4:4 (2003) E63.
[32] S. Ohtake, C. Schebor, S.P. Palecek, J.J. De Pablo, Phase behavior of freeze-dried phospholipids–cholesterol mixtures stabilized with trehalose, Biochim. Biophys. Acta Biomembranes 1713 (2005) 57–64.
[33] M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of dye-binding, Anal. Biochem. 72 (1976) 248–254.
[34] T.J. Mosmann, Rapid colorimetric assay for cellular growth and survival- Application to proliferation and cytotoxicity assays, Immunol. Methods 65 (1983) 5.
[35] A. Gürsoy, E. Kut, S. Özkırımlı, Co-encapsulation of isoniazid and rifampicin in liposomes and characterization of liposomes by derivative spectroscopy, Int. J. Pharm. 271 (2004) 115–123.
[36] P.A. Bridges, KMG. Taylor, Nebulisers for the generation of liposomal aerosols, Int. J. Pharm. 173 (1998) 117–125.
[37] S. Anabousi, E. Kleemann, U. Bakowsky, T. Kissel, T. Schmehl, T. Gessler, W. Seeger, C-M. Lehr, C. Ehrhardt, Effect of PEGylation on the stability of liposomes during nebulisation andin lung surfactant, J. Nanosci. Nanotechnol. 6 (2006) 1–7.
[38] J. Guo, Q. Ping, G. Jiang, L. Huang, Y. Tong, Chitosan-coated liposomes: characterization and interaction with leuprolide, Int. J. Pharm. 260 (2003) 167–173.
[39] H. Takeuchi, Y. Matsui, H. Yamamoto, Y. Kawashima, Mucoadesive properties of carbopol or chitosan-coated liposomes and their effectiveness in the oral administration of calcitonin to rats, J. Controlled Release 86 (2003) 235–242.